

CytoFLEX nano Standard Operating Procedures



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Starting up the CytoFLEX nano

- 1 Check the waste and sheath containers.
 - If the waste container is full or almost full, unscrew the lid and place it in the appropriate holder on the right side of the fluidics cart. Empty the waste into the sink and reconnect an empty container.
 - If the sheath container is empty or low, replace it with a new sheath container (CytoFLEX Sheath Fluid, cat. no. B51503).
- 2 Turn on the PC and log into Windows (no password is needed).
- 3 Turn on the instrument with a long press on the power button until the light changes from blue to green.

Start the *CytExpert* software: double-click the icon and sign into your user account.
- 4 Click *System Startup* in the acquisition window (top left corner of the screen – system startup takes 6 minutes).
- 5 When the system startup is completed click *Close*.
- 6 Go to the *QC/Sensitivity Monitor* tab and click *Start QC/Sensitivity Monitor*.

- 7 Under *Task* (top left side of the screen),
 - Click QC (red box in Figure 1).
 - Select the correct lot number for the Scatterspheres and Fluorospheres (Green box in Figure 1).Click the arrows to select.

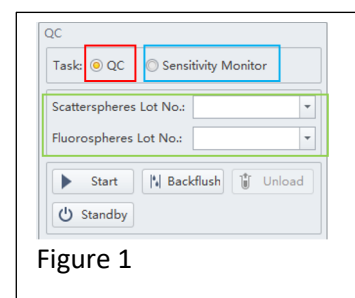


Figure 1

- 8 Click “►Start” to initiate the QC (if the instrument is in standby mode press  Initialize first. This only happens if the instrument is left for a longer period).

The QC process can be followed in the center-left part and the bottom of the screen. Initially, the instrument runs sheath fluid.

If the background is too high (*above 80 Events/Sec after the peak has been adjusted from a broad to a slim peak*), you will receive a warning. Then STOP the QC immediately (by pressing “■ Stop”) and perform the *Baseline Cleaning Cycle*

→ *Cytometer*

→ *Customized Baseline Clean*

→ Do not adjust the procedures in the pop-up window

→ Click *Start*. This takes 34 min.

- 9 Mix CytoFLEX nano QC Scatterspheres by gently turning the bottle upside down 10 times. It is important to be gentle to avoid bubbles!

Add 4 drops of beads into a 5 ml tube and place the tube in the sample holder.

- 10 Press *OK* when a pop-up asks for the Scatterspheres.

- 11 While the instrument is acquiring the QC Scatterspheres, you can prepare the QC fluorospheres; mix the beads by gently turning the bottle upside down 10 times. It is important to be gentle to avoid bubbles!

Add 3 drops into a 5 ml tube.

- 12 When the software informs you to change to QC fluorospheres change to this tube before pressing *OK*.

- 13 When QC is finished check the QC report, to make sure QC passed. If not, contact the FACS Core staff.

Setting up a new experiment

Important pre-experiment information

All samples **MUST** be filtered using a 0.45µm or 0.8 µm filter until FACS Core staff has given you permission to skip filtration.

Samples must be loaded in either 5 ml or Eppendorf tubes.

REMEMBER to remove the lid of 5ml tubes and unscrew/cut off the cap of Eppendorf tubes.

The instrument does NOT have a collision detector in the SIP.

The minimum volume to load is 100 µl (there is a large dead-volume of 32 µl).

If you are running beads in your experiment, dilute in filtered (0.02 µm) H₂O or sheath fluid.

Filtration must be performed daily through a 20 nm filter. Discard the first 10 ml filtered sheath to avoid particles from the filter.

VSSC1 roughly detects particles from 40nm-150nm, and VSSC2 particles from 80nm-1000nm.

Acquisition speed: Beads 1 µL/min, biological samples 2 µL/min

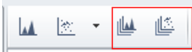
Use gloves!

Setting up a new experiment

- 1 Open CytExpert (if not already open), log in to your user profile, and select



(or System Startup) if the instrument is in Standby mode.

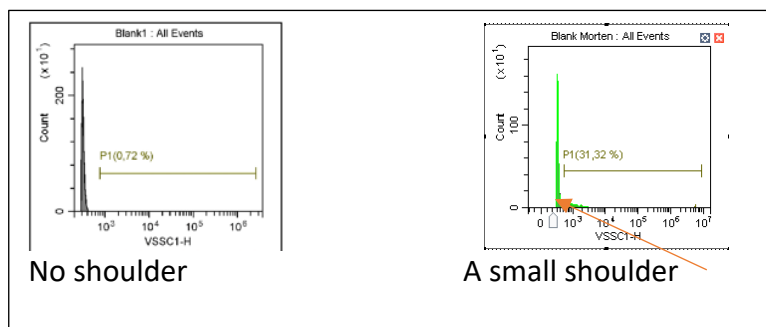
- 2 Go to **File** → **New Experiment** (or find a previous experiment).
- 3 Name your experiment and save it in a folder with your name.
- 4 Go to **Settings** → **Set Channel**. Uncheck the fluorescence channels you do not need. Under **Label**, type in the correct name of marker in the given detector. Press Apply and Close.
- 5 Create the plots covering your experimental setup. You can make multiple 1D or 2D plots at a time (Red box). . The image shows a toolbar with four icons: a histogram, a scatter plot, a 2D plot, and a 3D plot. The 2D plot icon is highlighted with a red box.
- 6 Create a VSSC1-H histogram for an inclusion gate and a VSSC1-Width vs VSSC1-H plot for doublet exclusion until you have determined the ideal sample dilution.
- 7 The threshold can be set to violet side scatter1 (VSSC1) and/or a fluorescence parameter. If you want a threshold only on a fluorescence parameter, you **MUST** initially determine the ideal sample dilution using VSSC1 as threshold. Otherwise, you risk a too high particle concentration (see *Nice to know in CytExpert*). Make sure to change the histogram recommended in step 6 for your threshold parameter.
- 8 Press **Acquisition Settings** to open a pop-up with three tabs: Gain, Threshold, and Width.

- a. *Gain*: adjust the gains settings according to your experiment. See *Nice to know in CytExpert* for more information about Acquisition settings.
- b. We recommend using the default settings when you start a new experiment (click the “Default” button in the pop-up window) but change gain for fluorescence detectors to 3000.
- c. *Threshold*: adjust the “Manual” value to 300 as a starting point.
- d. *Width*: If you want to exclude doublet using another parameter than VSSC1, it is possible, but NOT until you have determined the ideal sample dilution using VSSC1 as threshold.

9 Set Flow rate to slow (1µL/min).

10 Place a buffer sample in the sample loader (to rename the tube: right click the sample name and select Edit Name), and press “► Run”. A pop-up prompts you to **Set Sample Volume**. Type in your sample volume to avoid getting air into the instrument. The minimum volume to load is 100 µl. (There is a large dead-volume of 32 µl).

11 Check that your background is represented by a slim peak and no “shoulder” (if you have a shoulder go to step 17.)



12 Set a gate covering the area of the plot to the right of your background peak.

13 Press **Acquisition setting**. Change gain or threshold in the pop-up until you have about 20 events/sec in your blank buffer.

- a. You can also change the gain by drag and drop in a plot after clicking the  ikon. Similarly, you can adjust the Threshold in the plot using  : Move your cursor to the desired position for a threshold in a relevant histogram and click once.

14 Fill in *Events to Display* and *Time to Record*. Uncheck the *Volume to Record* and *Events to Record*.

15 When your settings are good, start recording data (Press **Record**). It is recommended to use Time as a stop condition.

16 Press **Unload**

- 17** If you have a shoulder in your plot in step 11 you must test whether the sample line is clean (if you are the first user, this step is included in the QC procedure):
- Press  to create a new blank sample. A Baseline Monitor window opens. Select Sample Line and type 100 in All events. (Do not add a sample). Press Apply and OK, and press “run”.
 - If more than 100 events are detected the instrument will clean and then test again until fewer than 200 events are detected unless you stop the process and select the On-board Clean to thoroughly clean the instrument (recommended after 1-2 attempts).
- 18** Press  to add a new sample. This sample will automatically have the settings from the previous sample.
- If you added samples before adjusting the gain and threshold on your previous sample, you must apply these settings to all other samples.
- 19** Your second sample should be your all-stained sample. Make a dilution series and start with the highest dilution → **The ideal concentration is $2 \times 10^6 - 10^8$ particles/ml**
- Rather start with a too-low concentration rather than a too-high concentration since the latter may cause high carry-over necessitating long cleaning procedures before you can continue.
- 20** Load the sample and check the event rate. If the event rate is above 10.000 stop immediately to avoid long cleaning procedures.
- Even at an event rate above 5000, you need to run a higher dilution. Theoretically, you can go higher, but it is not recommended. Remember to check the Processed Events (%) which should be 100% otherwise a higher dilution (lower concentration) is needed.
- 21** Once you have an appropriate event rate adjust the gain for the relevant fluorescence parameters if needed. Be aware that if you adjust, you must rerun your blank sample, since all samples in your experiment must be recorded with the same settings to be able to compare them.
- 22** For biological samples (not beads) you may adjust the acquisition speed to 2 µl/min. If you do this, do it for all samples and before you start recording.
- 23** If you suspect carryover between samples, check if the sample line is clean: Repeat step 10
- 24** Acquire your remaining controls and samples. Be aware of sample concentration and event rate.

25 Save an experimental template

26 Save a template if you would like to reuse the experiment later. See *Nice to Know in CytExpert*.

When you are finished acquiring your samples

- When you finish acquiring your samples, make sure to transfer your FCS files to your server and delete them on the PC afterward.
 - The FCS files are automatically exported to “This PC” and “Documents”.
C:\Users\“folder with own name”
- Transfer all files to OneDrive or the common server.
- Delete all your .fcs files from the PC.

If you need to change sheath while the instrument is turned on, Go to Cytometer, choose Standby first.

Compensation

- 1 If you need to compensate your samples first step is to save your gain settings:
 - a. To save acquisition settings from an experiment or a sample click the Acq. Settings icon and save either using “Export to Catalog” or “Export to File”.
- 2 Then go to **File → New compensation** – for automatic compensation.
- 3 A compensation Setup window appears. Select the relevant detectors and controls.
 - a. (If a negative population is not present in each single-color tube, an unstained control tube is needed e.g. unstained EVs. If you use compensation beads, you will have both positive and negative beads in your tube and therefore do not need an unstained control.
 - b. If you select “Sample” as Particle Type for your unstained control, select “Sample” for the corresponding single stained control. If you have two different unstained control types, select “Sample” for one of them and “Control” for the other (both unstained and single stained). Import the gain settings and apply the settings to all tubes.
 - c. If you want to include autofluorescence in your compensation matrix, you must include an unstained Sample.

Use	Tube	Label	Lot No.	Particle Type
☒	Unstained_Sample			☒ Sample ☐ Control
☐	Unstained_Control			☐ Sample ☒ Control
☒	V447			☐ Sample ☒ Control
☒	B531			☐ Sample ☒ Control
☒	Y595			☐ Sample ☒ Control
☒	R670			☒ Sample ☐ Control
☒	R710			☒ Sample ☐ Control
☒	R792			☒ Sample ☐ Control

☐ Select All

Dot Plot
 X-Axis: VSSC1
 Y-Axis: BSSC

OK Cancel

In this example, 3 samples will use the Unstained sample as “negative” and 3 control

- 4 Label and lot number information can be included in the Compensation Library to facilitate future compensation calculations.
- 5 Set the X-Axis and Y-Axis for your dot plot. The dot plot default setting is VSSC1 for X-Axis and BSSC for Y Axis.
- 6 Click OK.

- 7 Import the settings from your experiment: To import click the Acq. Settings icon and import either using "Import from Catalog" or "Import from File".
- 8 *The design of CytoFLEX nano system makes Gain independent compensation possible. CytExpert nano software automatically recalculates the compensation matrix according to gain. Manual adjustment may be applied but not necessary because it may introduce inaccurate results.*
- 9 Insert an unstained tube and select run to load the sample.
- 10 Set an appropriate number of particles to save in Events to Record.
- 11 Select Record to save the data.
- 12 Move the gate in the BSSC-H/VSSC1-H plot so that it encloses the desired population.
- 13 Acquire the remaining compensation samples like you would acquire your samples.
- 14 Check all acquired sample tubes and confirm that the gating is appropriate.
 - a. If your negative population is defined by an unstained sample, adjust the positive gate in each of the single-color stained samples, if necessary.
 - b. If your negative population is included in your single stained sample, adjust both the positive and the negative gate if necessary.
- 15 Select  or select Settings > Compensation Calculation to calculate the compensation values.
- 16 The Compensation Matrix window appears, displaying the calculated compensation values.
- 17 The Show Autofluorescence checkbox displays the autofluorescence.
- 18 The Use checkbox displays the effect of compensation on the highlighted sample.
- 19 The compensation values can be either black (OK), Orange (requires attention), or red (the value is > 400 or < -100, which is unacceptable).
- 20 Select Save As to export as a comp file and specify where to save it or select Save to Compensation Library to save the single-color compensation values in the compensation library. Remember to include keywords like date or lot number.
- 21 Save and close this compensation experiment and open your original experiment.
- 22 Highlight a sample and click Settings-> Compensation Matrix. Select Import and locate the path where your compensation matrix file (.comp) is saved to import the compensation values.
 - a. You can also select Import from Library to import compensation values from the compensation library. The Import from Compensation Library window

appears. From here you can select single colors to include in your compensation matrix.

- 23** Select which samples you want to apply the compensation to.
- 24** Select Import compensation matrix and convert based on the current gain.
- 25** Click apply to and select the samples you wish to compensate.
- 26** A grid will now be displayed next to compensated samples.
- 27** Click close.

User change on the CytoFLEX Nano

- 1 Check iLab to find out if someone is using the instrument after you.
- 2 Call the next user to make sure they will arrive as planned. If you are unable to reach that user:
 - a. within normal work hours inform the FACS Core staff.
 - b. outside normal work hours, perform the instrument shutdown (see that procedure).
- 3 For user change: Go to *Cytometer* and choose *On Board cleaning*.
- 4 When the cleaning is done go to *Cytometer*, choose *Standby*, and log out (upper right corner) of the CytExpert window.
- 5 Close CytExpert.
- 6 Remember to adjust your time in iLab and transfer your FCS files to your drive/server.

Turning off the CytoFLEX Nano

1 Check iLab to make sure no one booked the instrument after you today.

2 Go to *Cytometer*

→ *System Shutdown*

→ Select long-term soaking with cleaner.

This will take around 8 hours, but it is possible to skip the procedure after approximately 2 min.

3 You cannot log out of CytExpert or Windows until the procedure is finished.

Therefore, Press the  and **L** on the Keyboard. This will lock the screen, and no one will be able to access it without your log-in information.

4 Remember to adjust your time in iLab and to transfer your FCS files to your OneDrive or server.

Experimental Considerations

We recommend that the buffer you use is freshly filtered (daily) using a 0.02µm PES filter. Test the background in this buffer before you dilute your samples.

The following samples must be acquired for each experiment/sample

- Buffer only
- Dilution controls for your all-stained sample* – start with the lowest concentration (if MFI for one of your fluorescence parameters increases with increasing EV concentration you no longer have single EVs)
- Buffer + reagent – no EVs/particles in these samples, to be acquired at the same dilution as your EV sample.
- Unstained EVs (same dilution as the dilution of your all stain sample)
- Detergent control (for material that can be dissolved like EVs, LNPs etc.).

e.g. Triton-X 100 (Triton is diluted in water) added to a final concentration of 0.1% Triton-X 100 in your sample.

Incubate for 30 min. (Concentration and incubation time may vary depending on the sample).

Be aware that a higher event rate may be observed. If this is the case, you must dilute.

The following control samples is recommended to be acquired during experiment optimization

Besides the above-mentioned control samples, the following controls must be included during experiment optimization:

- Single-stained EV samples.
- Procedural controls – a sample from each step in your procedure to identify steps in the protocol that may introduce “false signals”.

Be aware that tips, tubes and filters may release particles that will increase your background.

*: unless you know you have the exact same concentration of EVs for each experiment.

Nice to know in CytExpert

Acquisition settings

We recommend using the default settings when you start a new experiment (click the "Default" button in the pop-up window). Please check the SSC tests located on the instrument lid.

If you want to import setting from a previous experiment, click either Import from Catalog or Import from File, depending on how you have saved your data.

If you want to **import** QC gains or from the sensitivity test, you must do it before you start running sample.

To **save** acquisition settings from an experiment you can save them either using "Export to Catalog" or "Export to File".

Use an experiment template

Create a new experiment from a template:

Select File -> New Experiment from Template. Select Browse next to New Experiment and specify the file path for the new experiment, then select Browse next to Template and specify the file path to the existing template.

Or

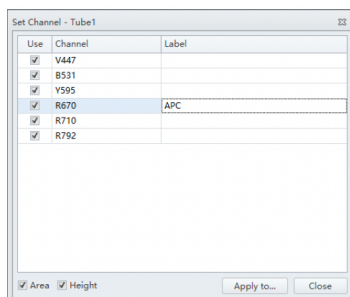
Select File -> New Experiment from Template, specify the file path and save the experiment.

Renaming your sample

To rename your sample right-click on the sample and choose Edit Name or double click on your sample name. Sample name will be included in your file name. Sample ID can be used to name sample groups, but will not be transferred to your FCS files.

Label your channel with reagent name

Select Setting -> Select Channel. Select the channel signal check box, then you can add the reagent name in the Label column. The information you add appears in the corresponding axis of the relevant plot in the plot area. Unselected channel signals are not stored in the data file.



Once all your labels are added, select Height and/or Area and click Apply to. In the new window, select which tubes this should be applied to.

Change parameter and population to show in a plot

Left click on the axis name to get a list of available parameters.

Left click in the upper part of the plot to select which population to include in the plot.

Change the appearance of your acquisition screen and plots

Select Settings -> Options

This enables you to define a default save path, define the columns displayed in the tube section and the appearance, you your plots including axis range and your page setup.

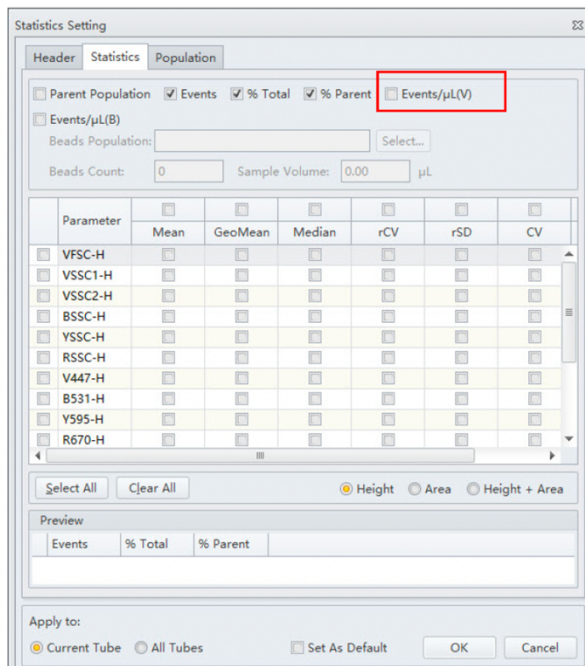
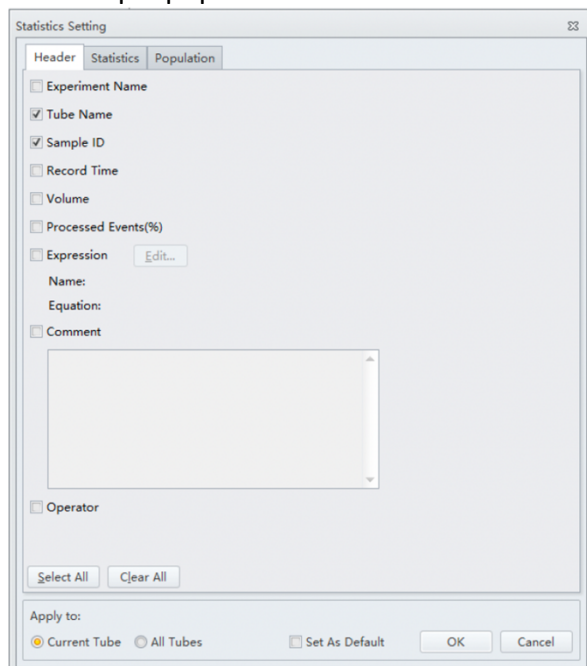
Do not disable Sample Volume check!

Add statistics

To add statistics in your plot area; click the statistics icon (Red box below)



If you want to add information to this window: Right-click on the statistics table and select Statistics Setting. This will allow you to change the display of the header, statistical elements and sample populations included.



Changing the sheath fluid, cleaning reagent, or the waste container while the instrument is turned on

Select Standby

Once the instrument has entered standby (the power button becomes blue), remove the tubing from the sheath container and replace the empty sheath fluid container with a new one. Since the old sheath container is not completely empty, transfer the lid from the new sheath container to the old container, write *Sheath* on the container and place it on the shelf.

Press Initialize in the software.

After cleaning reagent replacement:

Go to Cytometer -> perform *Piston Pump Debubble* twice.

After sheath fluid replacement:

Go to Cytometer -> perform *Sheath Filter Debubble* twice.

Go to Cytometer -> Select *Flow Cell Debubble*.

Save a PDF

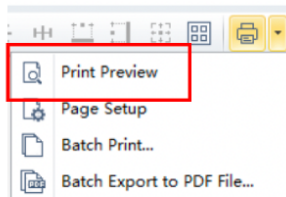
FACS Core Facility, Aarhus University

15/22

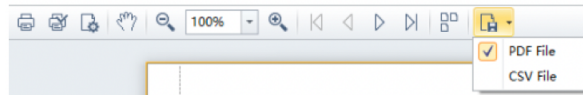
Last modified: 24.04.2026

You can save the plots and statistics in the *plot area* by converting them into .jpg or .pdf files.

Select the printer icon in the printer control area to print directly. Or select the print drop-down arrow for the following options:



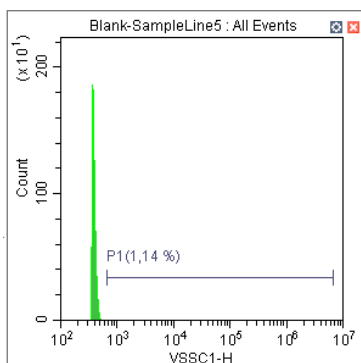
Select the format you wish to export to



If you have several samples, you can select the Batch Print or Batch Export to PDF File...

Scatter Calibration CytoFLEX nano

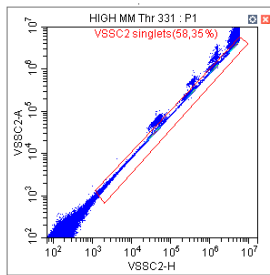
- 1 Run QC beads and make sure the instrument is clean.
- 2 Run Sensitivity Monitor.
- 3 Click start.
- 4 Perform sample line check: add a new blank  and make sure the instrument is clean.
 - a. Press  to create a new blank sample. A Baseline Monitor window opens. Select Sample Line and type 100 in All events. (Do not add a sample). Press Apply and OK, and press “run”.
 - b. If more than 100 events are detected the instrument will clean and then test again until fewer than 200 events are detected unless you stop the process and select the On-board Clean to thoroughly clean the instrument (recommended after 1-2 attempts).
- 5 Go to Acquisition settings and make sure to use the same settings as for the samples you are to calibrate.
- 6 Acquire your buffer, to be able to set an inclusion gate (P1 in the graph below).



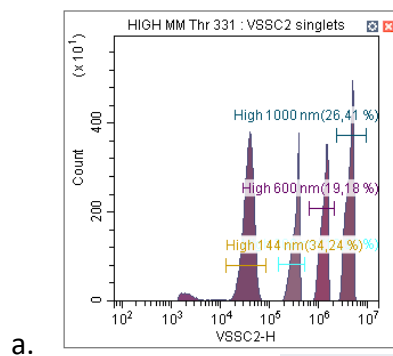
In the box with NanoVis beads, there are two vials one contains the nanoVis High beads (144 nm, 300 nm, 600 nm, and 1000 nm) and the nanoVis Low beads (44 nm, 80 nm, 105 nm, and 144 nm). BE AWARE that these are approximate sizes, the correct sizes are lot specific.

- 7 Mix the nanoVis High beads by gently turning the vial upside down 10 times and add 3 drops to a 1,5 ml tube (3 drops constitute less than 100 μ l, but is sufficient for your data collection, just type in 100 μ l in the software to be able to run).
- 8 Load and acquire the nanoVis High beads for 1-2 minutes.

9 Gate on singlets in a plot containing the events in the *inclusion gate*



10 Create a histogram of your singlets with VSSC2-H on the x-axis and create strict gates on peaks corresponding to 144 nm, 300 nm, 600 nm, and 1000 nm beads:



11 Create a statistics table

12 Right click anywhere in the statistics window and select Statistics Setting. This will open a new window with three tabs (Header, Statistics, and Population). Go to the Statistics tab and select Median for VSSC2-H. Go to the Population tab and select the relevant populations. Click Apply to “relevant samples” -> OK

FCM_{PASS} Half-angle determination and VSSC2 scatter calibration (for particles detected on in VSSC2)

13 Open the FCM PASS software. If you do this on the CytoFlex nano PC, you can use the login and password already typed in. If you do it on your own PC, you must register.

14 If you use FCM PASS on the CytoFLEX nano PC, you can skip this step. Otherwise, you must click Catalogue:

- Under Cytometers you must the instrument: register both the Cytometer ID (BH20023), Manufacturer (Beckman Coulter), and Model (CytoFLEX nano).
- Under Light Scatter you must register the beads you are using. A laminated document with this lot specific information will be located in the windowsill next to the CytoFlex nano.

15 If you use FCM PASS on the CytoFLEX nano PC this information is already typed in as the following data sets: NanoVis all (this dataset contains both Low and High beads), NanoVis Low (only contains the Low beads), NanoVis High (only contains High beads).

- 16 Close the FCM PASS Catalogue window.
- 17 Click Experiment Calibrator
- 18 Select your instrument under Cytometer ID
- 19 Click the “+” next to Datasets to create a new dataset. Insert the information needed in the popup window and click Save.
- 20 Go to the “1. File Import tab”, click the “+” next to Files to Calibrate and locate the path to the folder with your fcs files. You can import both the NanoVis High and Low files and your actual samples.
- 21 Under sample type in the second column, click the arrow to the right for the NanoVis High samples and select “SSC Calibrator” instead of “Sample”

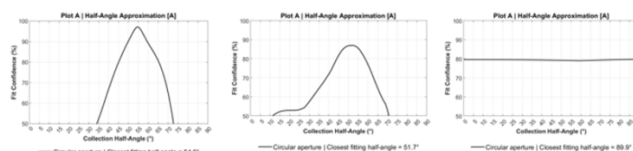
NanoVis High.fcs	Sample
NanoVis Low.fcs	SSC Calibrator

- 22 Click “Next” to enter the Fluorescence Calibration tab and “Next” again to enter the Scatter Calibration tab.
- 23 Click the “+” next to Side Scatter Calibration. Go to Bead Set -> click the arrow and

+ - Side Scatter Calibration					
Selection	Scatter Parameter	Scatter Wavelength (nm)	Scatter Threshold	Bead Set	Sheath RI
☒	SSC_1-H VSSC2-H	405	NA	All	1.3431

select NanoVis High.

- 24 The list of beads populations now appears in the lower part of the window. In the Column Acquired Stats, you must now type in the VSSC2-H Median value for the corresponding population from your NanoVis High sample.
- 25 Click calibrate when ready and wait for the confirmation prompt.
- 26 A new window of the folder containing calibrated data along with the FCMPASS Figure, Encrypted_Report.exRNA, and FCMPASS REPORT.xlsx.
- 27 Open and review the FCM_{PASS} Figure 1.png
 - a. In plot A, locate the Closest fitting half angle and check the corresponding %Fit Confidence and the closest fitting half-angle (The peak of the curve). The Fit confidence must be >90%. If not, go back and check that QC passed, and that your gates and median values are correct and from VSSC2-H.



Acceptable Half-angle

- > 95% Fit confidence of peak half-angle
- Sharp, narrow peak suggesting higher certainty of half-angles

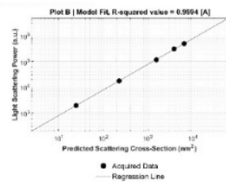
Review Accuracy of Data

- < 90% Fit confidence of peak half-angle
- Broad peak especially on the extremes suggesting uncertainty.

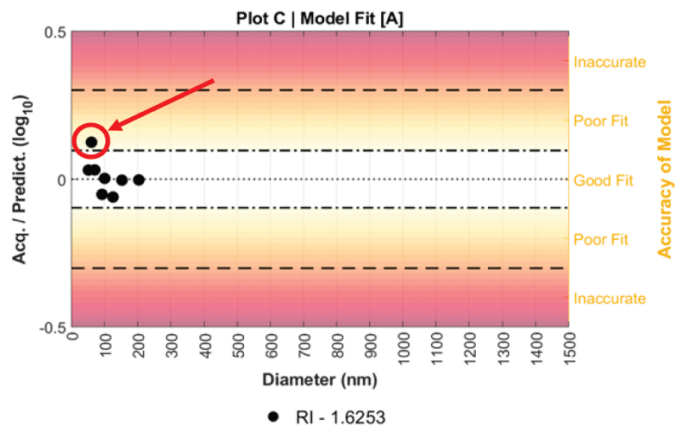
Review Accuracy of Data

- Check QC report for misalignment in violet channel
- > Poor fit confidence
- Flat line from 0° to 90° high uncertainty of half-angles

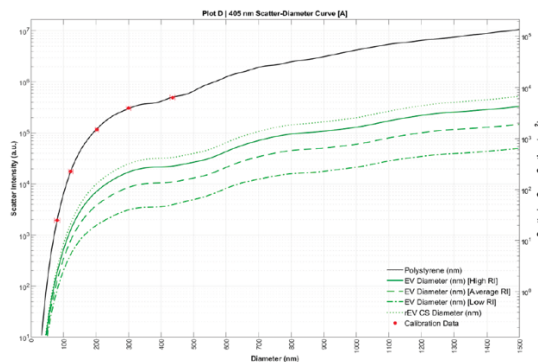
- i.
- b. Check plot B if a reference particle does not match the correlation, check that the median data has been entered correctly, and gates are set strict.



- i.
- c. Check plot C to see if the collected median data of each bead size compares to the predicted value. If a particle is found in the regions of Poor Fit or Inaccurate, check that the median and gates.



- i.
- d. Check plot D. If any discrepancies are found. Review that the data has been inputted correctly.



e. **Figure 27.** Representative Plot D 405 Scatter-Diameter Curve

28 Note down the half angle approximation value for calibrating the VSSC1 light scattering channel.

FCM_{PASS} VSSC1 scatter calibration using half-angle approximation from VSSC2 (for particles detected on in VSSC1)

- 29** Before performing the VSSC1 scatter calibration ensure that
- QC passed.
 - Sensitivity monitor passed.
 - Half-angle determined by VSSC2 hat a fit Confidence >90%.

- d. Baseline monitor shows CytoFLEX nano instrument is clean.
 - e. Samples have been acquired at an ideal sample dilution (to avoid swarming).
 - f. NanoVis Low beads have been acquired after all the above at the same settings as the samples you would like to calibrate.
- 30** Mix the nanoVis Low beads by gently turning the vial upside down 10 times and add 3 drops to a 1,5 ml tube.
 - 31** Load and acquire the nanoVis Low beads for 1-2 minutes.
 - 32** Gate on singlets in a plot containing the events in the *inclusion gate* in a VSSC1-A vs VSSC1-H plot.
 - 33** Create a histogram of your singlets with VSSC1-H on the x-axis and create gates on peaks corresponding to 80 nm, 105 nm, and 144 nm beads. Find the VSSC1-H median for these populations.
 - 34** Click “Experiment Calibration” and create a new dataset by selecting the cytometer (BH20023).
 - 35** Click the “+” icon. Enter date, dataset name and Dataset notes.
 - 36** Save dataset
 - 37** Click “Begin Calibration”
 - 38** Under File Import, click the “+” to select the folder containing your fcs. files to be calibrated including the NanoVis Low.
 - 39** In the Sample type column, select SSC calibrator for the NanoVis files.
 - 40** Click Next
 - 41** Click next again to skip fluorescence calibration and go to scatter calibration.
 - 42** Click the “+” to add a selection
 - 43** Choose SSC-H | VSSC1-H in the drop-down in the second column.
 - 44** Select the NanoVis Low beads set under beads set.
 - 45** Enter the VSSC1-H median data for the 80 nm, 105, and the 144 nm beads into the acquired stats column (if multiple gains settings were used, create separate calibration for each of the different gains).
 - 46** Click the “Advanced Settings” towards the bottom of the window. A new window appears.
 - 47** Select the “Modelling Parameters” tab.
 - 48** Under the determine Half-Angle column, turn off “determine half-angle”. New columns will now appear: Theta, Phi, and Epsilon. Epsilon is the half-angle. Enter in 90 for both the Theta and Phi (orientation angles of the flow cell) and the approximated half-angle determined for VSSC2 under the epsilon column.
 - 49** Return to the “Particle Modelling” tab. The table shows several theoretical built-in Core-shell Parameter RI.

- 50** It is possible to add additional Core-shell channels in nanometres for RI determined for your samples. Click “+” to add and remember to add a unique name for the custom RI.
- 51** By default, the FCM_{PASS} provides RI calibration parameters specifically for EVs using a core-shell model with a shell thickness of 5 nm and three RI values ranging from low RI of 1.3658 to high RI of 1.4060 to model a heterogenous distribution of EVs. rEV refer to Sigma-Aldrich Exosome standards, fluorescent PN: SAE0913.
- 52** Close the Advanced setting window to save the inputted parameters.
- 53** Click Next and then Calibrate.
- 54** Open and review the FCM_{PASS} Figure 1.png
- 55** Be aware that Plot A is missing because a known half-angle was used.
- 56** Check that the median values entered demonstrate a good fit of the model in Plot B, C, and D.